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Identification and Molecular Characterization of Foot and Mouth Disease Virus Based on VP1 Gene Fragments in Madura Cattle and Ongole Grade Cattle

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ABSTRACT

Foot and mouth disease (FMD) is an infectious vesicular disease of cloven-hoofed animals caused by the FMD virus. It is acute, highly contagious, and has a lot of genetic diversity. The aim of this study was to confirm cases diagnosed in the field as FMD virus (FMDV) infection through identification and molecular characterization based on the amplification of the VP1 gene of FMDV to provide information about serotype, virus clustering, and additional molecular scientific data on FMDV circulating in Indonesia. The samples used in this study were Madura cattle and Ongole Grade cattle, which showed clinical signs of FMD. Twenty-six samples were collected from the vesicular fluid of blister epithelial cells (tongue, gum, and hard palate), oral, and nasal swabs. Those samples underwent a screening test using the real-time reverse transcription-polymerase chain reaction (RT-qPCR) method with a 3D gene target to detect FMDV infection. About 46.15% of samples (12/26) were detected as RT-qPCR positive for FMDV. Those positive results were then amplified by reverse transcription-polymerase chain reaction (RT-PCR) and sequenced using the Sanger sequencing technique targeting the VP1 gene fragment of the FMDV. The sequencing results were analyzed by the Molecular Evolutionary Genetics Analysis (MEGA) software X version, which includes assembly, alignment using ClustalW, amino acid prediction, genetic distance, and phylogenetic tree construction. The result showed that amino acid sequence variations were found in this gene, including at positions 96, 99, 129, 134, 138, 140, 156, 158, and 197, and no changes were found either at the critical amino acid sites at positions 144 (V), 148 (L), 154 (K), and 208 (P) or in the arginine-glycine-aspartic acid (RGD) motif at positions 145–147. Phylogenetic analysis indicated that FMD viruses detected in this study were identified as serotype "O", topotype "Middle East South Asia (ME-SA)", lineage "Ind-2001", and sub-lineage "e" (O/ME-SA/Ind-2001e), which have high homology to the VP1 gene (99–100%) between the viruses studied and the viruses found at the beginning of the FMD outbreak in Indonesia in 2022.

Keywords: foot and mouth disease; molecular characterization; phylogenetic analysis; serotype O; VP1 gene

INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious viral disease. Although a disease of low mortality, the global impact of FMD is very significant. Direct losses due to this disease cause a decrease in the quality and quantity of livestock, while indirect losses cause a decline in the economy due to the costs of controlling and treating the disease (Knight-Jones & Rushton, 2013). FMD was first reported by Hieronymus Fracastorius in 1514 in Venice, Italy (Jamal & Belsham, 2013). This disease was first reported in Indonesia in 1887. Indonesia has been successfully declared an FMD-free country by the OIE since 1990, but an outbreak of FMD reoccurred on April 28, 2022. The FMD outbreak first appeared in several areas in East Java, such as Gresik, Lamongan, Sidoarjo, and Mojokerto. As of October 25, 2023, FMD was still spreading in 19 provinces and 125 districts/cities, with the number of infected cattle reaching 615,570 heads (DGLAHS, 2023).

FMD is caused by the FMD virus (FMDV), which belongs to the *Aphthovirus* genus of the *Picornaviridae* family (OIE, 2022). The FMDV consists of an RNA genome surrounded by a capsid. The capsid is composed of 60 copies of the capsomers, where each capsomere contains four structural viral proteins (VP):

VP1 (1D), VP2 (1B), VP3 (1C), and VP4 (1A), which self-assemble into a spherical icosahedron with a diameter of approximately 25 to 30 nm and no lipid envelope. The structural proteins VP1, VP2, and VP3 are exposed on the capsid surface, while VP4 is entirely inside the capsid (Jamal & Belsham, 2013; Reeve *et al.*, 2016). FMDV genome is a single positive strand chain of about 8400 nucleotides (nt) and encodes a large polyprotein from a single open reading frame (ORF) of about 7000 nt. The uncapped viral RNA is flanked by a very long (~1300 nt) 5′ untranslated region (5′ UTR) and a short (~90 nt) 3′ untranslated region (3′ UTR), and ends with a polyadenylation or poly(A) tail (Belsham & Botner, 2015). The ORF can be translated into polyproteins, then processed by viral proteases to form four structural proteins (VP1, VP2, VP3, and VP4); ten non-structural proteins (L^{pro}, 2A, 2B, 2C, 3A, $3B_{1-3}$, $3C^{pro}$, and $3D^{pol}$); and some precursors that have different functions (Gao *et al.*, 2016).

There are seven serotypes of FMDV (O, A, C, Asia 1, and South African Territories (SAT) 1-3), where FMDV infection caused by one virus serotype does not confer immunity against the other serotypes. The virus serotypes are not distributed uniformly worldwide, where this disease still occurs. In the African region, six FMDV serotypes were found (O, A, C, SAT 1, SAT 2, and SAT 3). In Asia, only four serotypes of FMDV were found (O, A, C, and Asia 1), while in South America and Europe, only three serotypes were found (O, A, and C) (Jamal & Belsham, 2013; OIE & FAO, 2012). In Southeast Asian countries, serotype O is the dominant serotype causing FMD outbreaks compared to serotypes A and Asia 1 (Brito *et al.*, 2017). The serotype O is divided into eleven topotypes known as East Africa 1 to 4 (EA-1 to -4), Southeast Asia (SEA), Europe-South America (EURO-SA), Indonesia-1 and -2 (ISA-1 and -2), CATHAY, Middle East-South Asia (ME-SA), and West Africa (WA) (Knowles *et al.*, 2016; WRLFMD, 2022).

The diversity of serotypes and limited data on the molecular characteristics of FMDV are challenges in controlling FMD in Indonesia; therefore, it is necessary to update data on the molecular characteristics of FMDV. The VP1 protein is exposed on the surface of the virus capsid and is the main protein that can be used to determine the serotype and genotype of FMDV (Liu *et al.*, 2017). The results of this research are expected to confirm cases diagnosed with FMD, provide information about serotype prediction, virus clustering, and add molecular scientific data on FMDV in Indonesia. The aim of this study was to confirm the diagnosis of Madura Cattle and Ongole Grade Cattle as FMDV infections and molecular characterization based on the amplification of the VP1 gene fragment to predict the serotype of the detected FMDV.

MATERIALS AND METHODS

Ethical Approval

The samples were obtained from FMD cases on cattle farms. The research method has been reviewed and approved by the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia (approval number 3631/UN1/FKH.1/TU/PT/2023).

Study Period and Location

This research was conducted from July 2022 to September 2023 at the Microbiology Laboratory of the Faculty of Veterinary Medicine, UGM, Yogyakarta, Indonesia; the Biotechnology Laboratory of the Disease Investigation Center (DIC) Wates, Yogyakarta, Indonesia; and the National Center for Veterinary Biologics, Surabaya, Indonesia.

Samples

This research was conducted on 26 Madura cattle and Ongole Grade cattle that showed clinical symptoms of FMD. Madura cattle (n= 15) were male, while Ongole Grade cattle (n= 11) were female, 1.5 to 2 years old, and had never received the FMD vaccine. The research samples were collected from vesicular fluid from blister epithelial cells (tongue, gums, and hard palate), oral, and nasal swabs. The samples were then either stored in the viral transport medium (VTM) and shipped on ice or frozen cold packs to the receiving laboratory. Samples were collected in July 2022 from a cattle farm in the Boyolali District of Central Java Province suspected of being infected with FMDV based on the clinical symptoms encountered.

FMDV Detection

The FMDV was detected using real-time reverse transcription-polymerase chain reaction (RT-qPCR) by following established procedures and referring to OIE Terrestrial Manual FMD guidelines. Viral RNA was extracted from 26 samples using the Pure Link™ Viral RNA/DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The reagent mix for RT-qPCR consisted of SensiFAST™ Probe Lo-ROX One-Step Kit (Bioline Reagents Ltd., London, United Kingdom), a specific primer set (forward, reverse, and probe primers), and DEPC-treated water that was added to the extracted viral RNA. The reaction mix was performed according to the manufacturer's instructions. Primers used for RT-qPCR were one set of primers-probes for the 3D gene of FMDV (Invitrogen, Thermo Fisher Scientific, USA) listed in Table 1. Negative and positive controls were used in each run to ensure successful amplification. The solution was amplified using an ABI 7500 Fast Real-time PCR System machine (Applied Biosystems), with the following settings: reverse transcription (45 °C for 10 minutes) for 1 cycle; polymerase activation (95 °C for 10 minutes) for 1 cycle; denaturation (95 °C for 15 seconds), and annealing/extension (60 °C for 45 seconds) for 45 cycles. The RT-qPCR results appeared on the monitor screen in the form of curves and cycle threshold (C_T) values. The C_T value of < 40 was considered positive, the C_T value of 40 – < 45 was considered indeterminate or dubious, and the C_T value = 45 was considered negative (OIE, 2022; Reid *et al.*, 2001).

Table 1. Primers set for RT-qPCR (3D gene) and RT-PCR (VP1 gene) of foot and mouth disease virus

Primer	Sequence 5'-3'	Amplicon size	Reference
FM3D-F	ACTGGGTTTTACAAACCTGTGA	107 bp	(Callahan *et al*., 2002)
FM3D-R	GCGAGTCCTGCCACGGA		
FM3D-Probe	TCCTTTGCACGCCGTGGGAC		
UNI-VP1F	AGYGCYGGYAARGAYTTTGA	821 bp	(Le *et al*., 2012)
UNI-VP1R	CATGTCYTCTYGCATCTGGTT		

Note: RT-qPCR= real-time reverse transcription-polymerase chain reaction; RT-PCR= reverse transcription-polymerase chain reaction.

Amplification and Sequencing of the VP1 Gene of FMDV

The samples that showed positive for FMDV results from the RT-qPCR assay were then subjected to a reverse transcription-polymerase chain reaction (RT-PCR) to obtain PCR products for further sequencing. Viral RNA was extracted using the Viral Nucleic Acid Extraction Kit II (Geneaid Biotech Ltd., New Taipei, Taiwan) according to the manufacturer's instructions. The RT-PCR method is performed in two-step assays, where reverse transcription (RT) and polymerase chain reaction (PCR) steps are performed in separate tubes. The reagent mix for RT consisted of SensiFAST™ cDNA Synthesis Kit (Bioline Reagents Ltd., London, United Kingdom), DEPC-treated water, and the extracted viral RNA. The reaction mix was performed according to the manufacturer's instructions. The solution was amplified using a T100™ Thermal Cycler machine (Bio-Rad Laboratories) with the following settings: primary annealing (25 °C for 10 minutes), reverse transcription (42 °C for 15 minutes), and inactivation (85 °C for 5 minutes). The RT result is a cDNA template that will be used for the PCR process.

The reagent mix for PCR consisted of MyTaq™ HS Red Mix (Bioline Reagents Ltd., London, United Kingdom), a set of specific primers (forward and reverse primers), DEPC-treated water, and the cDNA template resulting from RT. The reaction mix was performed according to the manufacturer's instructions. The primers used for RT-PCR were a set of primers targeting the VP1 gene of FMDV (Invitrogen, Thermo Fisher Scientific, USA) listed in Table 1. The solution was amplified using a T100™ Thermal Cycler machine (Bio-Rad Laboratories) with the following settings: initial denaturation (95 °C for 5 minutes) for 1 cycle; denaturation (94 °C for 1 minute), annealing (56.5 °C for 1 minute), and extension (72 °C for 2 minutes) for 40 cycles; and final extension (72 °C for 10 minutes) for 1 cycle.

The PCR product was confirmed by electrophoresis on 1.2% of agarose gel in 1X TBE (trizma base-boric acid-EDTA) buffer, FloroSafe DNA Stain (1st Base, Singapore) as a staining agent, and using GeneRuler 100 bp DNA Ladder (Thermo Scientific, USA) as a size marker. The electrophoresis was visualized using a gel documentation/high-performance ultraviolet transluminator (UVP) to see the DNA band due to the amplification of the VP1 gene of the FMDV. A positive result is indicated by forming a band at position 821 bp, and a negative result is indicated if there is no band on the gel. The PCR products from samples that show positive electrophoresis results are sequenced using the Sanger Sequencing System method (Sanger Sequencing Services, 1st Base, Selangor, Malaysia).

Phylogenetic Analysis

The sequencing obtained based on the VP1 gene of FMDV was analyzed and implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software X version (Kumar *et al*., 2018), which includes assembly, alignment using ClustalW, amino acid prediction, genetic distance, and phylogenetic tree construction. The relationship between the FMDV sample was compared with reference isolates downloaded from the GenBank of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed by the Neighbor-Joining method with 1000 bootstrap replicates using the Kimura two-Parameter model.

RESULTS

Clinical Signs of FMD

The present study was performed on 26 cattle exhibiting clinical signs of FMD. The animals showed hypersalivation; lesions formed on the coronary band, nose, gums, and hard palate; and vesicles formed on the dental pads, gums, and feet (Figure 1).

FMDV Detection

All samples were tested by RT-qPCR, which confirms the presence of FMDV infection based on the 3D gene. The results of FMDV detection using the RT-qPCR method on 26 samples: 12 samples showed positive results of 46.15% (12/26), with positive results on samples of Madura cattle of 40% (6/15) and Ongole Grade cattle of 54.54% (6/11). The results of RT-qPCR based on the 3D gene of FMDV are listed in Table 2, with amplification curves presented in Figure 2.

Amplification and Sequencing of the VP1 Gene of FMDV

Twelve samples that showed positive FMDV results from the RT-qPCR assay were then subjected to RT-PCR. The results of electrophoresis visualization from the RT-PCR assays based on the amplification of the VP1 gene of FMDV showed that 41.67% (5/12) samples were positive, the sample band was similar to the

Figure 1. Clinical signs of foot and mouth disease in Madura cattle (A, B, C) and Ongole Grade cattle (D, E, F) samples. A= hypersalivation; B= lesions on the coronary band; C= lesions on the nose; D= lesions on the gums and hard palate; E= vesicles on the dental pads and gums; F= vesicles on the feet.

positive control (K+) band, which appeared at position 821 bp. The band of the sample looked clean, bright, single, and without any extra bands of contamination. The results of RT-PCR based on amplification of the VP1 gene of FMDV are listed in Table 2, and agarose gel electrophoresis results are presented in Figure 3. The PCR products from five samples that showed positive electrophoresis results were sequenced.

Phylogenetic Analysis

Five sample sequences were obtained based on the VP1 gene of FMDV and then phylogenetically analyzed. The VP1 gene of the sample yielded 639 nucleotides, encoding about 213 amino acids. The VP1 gene sequence data for five isolates in this study were deposited in GenBank under accession numbers OR570805–OR570809 (Table 3). Sequence analysis of the sample in this study showed amino acid variations at positions 96, 99, 129, 134, 138, 140, 156, 158, and 197, and no changes were found either at the critical amino acid sites at position 144 (V), 148 (L), 154 (K), and 208 (P), or in the arginine-glycine-aspartic acid (RGD) motif at positions 145–147 (Table 4). The genetic distance between the sample and reference isolates from the GenBank data center can be seen in Table 5.

Table 2. The results of RT-qPCR and conventional RT-PCR of the sample collected from vesicular fluid from the blister epithelial cells (tongue, gums, and hard palate), oral, and nasal swabs of Madura cattle and Ongole Grade cattle which were stored in deep freezer

No.	Sample code	Breed	RT-qPCR		RT-PCR
			C_T value	Interpretation	
1	MHW20	Ongole Grade Cattle	35.43	Positive	Negative
2	MHW32	Ongole Grade Cattle	30.38	Positive	Positive
3	MHW36	Ongole Grade Cattle	45	Negative	NA
4	MHW37	Ongole Grade Cattle	45	Negative	NA
5	MHW42	Ongole Grade Cattle	45	Negative	NA
6	MHW52	Ongole Grade Cattle	38.36	Positive	Negative
7	MHW68	Ongole Grade Cattle	39.24	Positive	Negative
8	MHW74	Ongole Grade Cattle	45	Negative	NA
9	MHW84	Ongole Grade Cattle	31.17	Positive	Positive
10	MHW87	Ongole Grade Cattle	29.86	Positive	Positive
11	MHW88	Ongole Grade Cattle	45	Negative	NA
12	MHW11B	Madura Cattle	45	Negative	NA
13	MHW108	Madura Cattle	45	Negative	NA
14	MHW124	Madura Cattle	45	Negative	NA
15	MHW126	Madura Cattle	45	Negative	NA
16	MHW128	Madura Cattle	45	Negative	NA
17	MHW145	Madura Cattle	27.32	Positive	Positive
18	MHW168	Madura Cattle	38.22	Positive	Negative
19	MHW184	Madura Cattle	45	Negative	NA
20	MHW219	Madura Cattle	35.39	Positive	Negative
21	MHW222	Madura Cattle	45	Negative	NA
22	MHW230	Madura Cattle	36.81	Positive	Negative
23	MHW249	Madura Cattle	37.42	Positive	Negative
24	MHW305	Madura Cattle	45	Negative	NA
25	MHW311	Madura Cattle	26.7	Positive	Positive
26	MHW360	Madura Cattle	45	Negative	NA

Note: RT-qPCR= real-time reverse transcription-polymerase chain reaction; RT-PCR= reverse transcription-polymerase chain reaction; C_T= cycle threshold; NA= not tested.

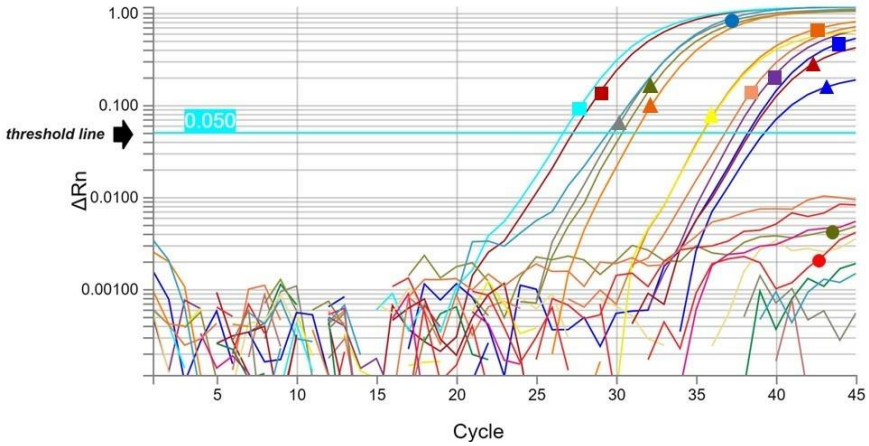

Figure 2. Amplification plots of the RT-qPCR assay based on the 3D gene of the collected field sample. The C_T value of < 40 was considered positive; the C_T value of 40 – < 45 was considered indeterminate or dubious; and the C_T value = 45 was considered negative with a threshold line of 0.05. Line= samples code (C_T value); ▲= MHW20 (35.43); ▲= MHW32 (30.38); ▲= MHW52 (38.36); ▲= MHW68 (39.24); ▲= MHW84 (31.17); ▲= MHW87 (29.86); ■= MHW145 (27.32); ■= MHW168 (38.22); ■= MHW219 (35.39); ■= MHW230 (36.81); ■= MHW249 (37.42); ■= MHW311 (26.70); ●= positive control; ●= negative control; ●= NTC. Sample code MHW20, MHW32, MHW52, MHW68, MHW84, and MHW87 are Ongole Grade cattle, while sample code MHW145, MHW168, MHW219, MHW230, MHW249, and MHW311 are Madura cattle.

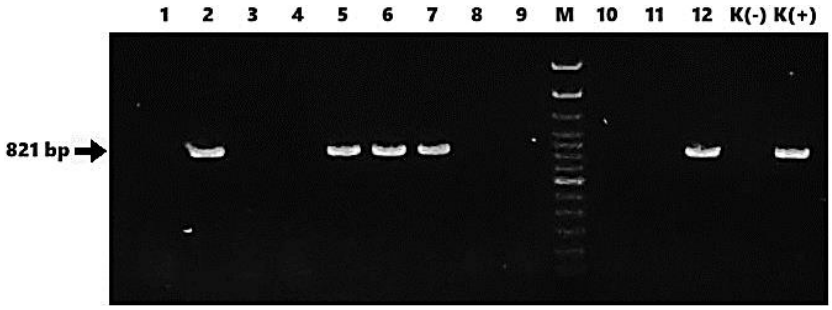

Figure 3. Agarose gel electrophoresis of RT-PCR products based on the VP1 gene of foot and mouth disease virus. PCR products measuring 821 bp. 1= MHW20; 2= MHW32; 3= MHW52; 4= MHW68; 5= MHW84; 6= MHW87; 7= MHW145; 8= MHW168; 9= MHW219; 10= MHW230; 11= MHW249; 12= MHW311; M= 100 bp DNA Ladder markers; K(-)= negative controls; K(+)= positive controls. Sample code MHW20, MHW32, MHW52, MHW68, MHW84, and MHW87 are Ongole Grade cattle, while sample code MHW145, MHW168, MHW219, MHW230, MHW249, and MHW311 are Madura cattle.

Table 3. List of samples with sequence results that have been deposited in GenBank originated from Madura cattle and Ongole Grade cattle (PO cattle)

No.	Sample code	Sample name	Accession number
1	MHW32	O/ISA/MHW32-POCattle/2022	OR570805
2	MHW84	O/ISA/MHW84-POCattle/2022	OR570806
3	MHW87	O/ISA/MHW87-POCattle/2022	OR570807
4	MHW145	O/ISA/MHW145-MaduraCattle/2022	OR570808
5	MHW311	O/ISA/MHW311-MaduraCattle/2022	OR570809

The phylogenetic tree analysis of the five samples in this study found them within the same subcluster as the reference isolates from Indonesia, with access numbers OP585403, ON854957, ON854956, ON854955, and ON854954. The results of the phylogenetic analysis are presented in Figure 4.

DISCUSSION

FMD outbreaks are a major animal health problem within Southeast Asia (SEA), causing enormous economic losses in affected countries where serotype O dominates throughout SEA (Blacksell *et al.*, 2019). FMD cases are characterized by clinical signs such as vesicular conditions of the feet, buccal mucosa, and mammary glands in female animals. Clinical signs can vary from mild to severe, and death may occur, especially in young animals (OIE, 2022). Typical clinical signs of FMD are characterized by fever, hypersalivation, vesicles in the mouth, nose, interdigital space, and coronary band (Jamal & Belsham, 2013). The clinical signs shown in the cattle in this study were similar to those of FMD (Figure 1). The collected samples were vesicular fluid from blisters (tongue, gums, and hard

Table 4. Amino acid variations based on the VP1 gene of foot and mouth disease virus in this research were compared with reference isolates from GenBank

No.	Isolate name/ FMDV strain	Amino acid positions															
		96	99	129	134	138	140	**144**	145	146	147	**148**	**154**	156	158	197	**208**
1	*O1/Manisa/TUR/69* (*AY593823*)	*A*	*D*	*V*	*S*	*D*	*T*	**V**	*R*	*G*	*D*	**L**	**K**	*A*	*A*	*D*	**P**
2	O/LAO/2/2006 (EU667451)	T	.	.	C	E	P	**.**	.	.	.	**.**	**.**	.	T	S	**.**
3	O/IRN/31/2009 (KY091284)	T	.	.	C	E	H	**.**	.	.	.	**.**	**.**	.	T	A	**.**
4	O/IRN/18/2010 (KY091283)	K	.	.	C	E	H	**.**	.	.	.	**.**	**.**	.	T	S	**.**
5	O/IRN/88/2009 (KY091282)	T	.	.	C	E	S	**.**	.	.	.	**.**	**.**	.	T	N	**.**
6	O/PAK/16/2010 (KY091285)	T	.	.	C	E	Q	**.**	.	.	.	**.**	**.**	.	T	N	**.**
7	O/KUW/3/97 (DQ164904)	T	.	.	C	E	A	**.**	.	.	.	**.**	**.**	.	T	S	**.**
8	O/OMN/7/2001 (DQ164941)	K	.	.	C	E	A	**.**	.	.	.	**.**	**.**	.	T	N	**.**
9	O/UAE/4/2008 (KM921876)	T	.	.	C	.	V	**.**	.	.	.	**.**	**.**	.	.	S	**.**
10	O/BHU/3/2009 (KM921814)	.	.	.	C	E	D	**.**	.	.	.	**.**	**.**	.	T	S	**.**
11	O/ISA/1/2022 (OP585403)	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	.	E	**.**
12	O/ISA/HSU/A0522099-5/2022 (ON854957)	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	.	E	**.**
13	O/ISA/Magetan/A04222620/2022 (ON854956)	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	.	E	**.**
14	O/ISA/Semarang/A04222614/2022 (ON854955)	T	E	.	C	E	A	**.**	.	.	.	**.**	**.**	T	.	E	**.**
15	O/ISA/Pemalang/A04222613/2022 (ON854954)	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	.	E	**.**
16	O/CAM30/2019 (MZ634456)	.	.	.	C	E	A	**.**	.	.	.	**.**	**.**	.	T	E	**.**
17	O/HKN/21/70 (AJ294911)	T	.	.	C	.	H	**.**	.	.	.	**.**	**.**	S	.	S	**.**
18	O/TAI/189/87 (KY091288)	T	.	.	C	E	S	**.**	.	.	.	**.**	**.**	.	P	S	**.**
19	C/N65/Tadjikistan/USSR/67 (KY091302)	S	.	A	T	S	-	**R**	S	.	.	**M**	**A**	.	R	T	**.**
20	Asia1/PAK/1/54 (AY593795)	T	.	.	T	E	-	**M**	.	.	.	**R**	**.**	N	Q	T	**.**
21	O/ISA/MHW32-POCattle/2022 (OR570805)*	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	T	E	**.**
22	O/ISA/MHW84-POCattle/2022 (OR570806)*	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	T	E	**.**
23	O/ISA/MHW87-POCattle/2022 (OR570807)*	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	T	E	**.**
24	O/ISA/MHW145-MaduraCattle/2022 (OR570808)*	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	.	E	**.**
25	O/ISA/MHW311-MaduraCattle/2022 (OR570809)*	T	E	A	C	E	A	**.**	.	.	.	**.**	**.**	T	.	E	**.**

Note: * (asterisk)= samples; - (dashes)= gaps; . (dots) = amino acid similarities; bold characters= the most critical amino acids in the VP1 protein epitope of the FMD serotype O virus are 144 (valine), 148 (leucine), 154 (lysine), and 208 (proline); grey highlighted regions= the conserved arginine (R), glycine (G), and aspartic acid (D) (RGD) motive is at amino acid positions 145–147.

palate), oral and nasal swabs. In clinically affected animals, the preferred samples are materials from the lesions (OIE, 2018). Suspected cases of FMD can be identified based on the observation of clinical signs. The severity of symptoms in animals is influenced by many factors, such as the type of animal, age of the animal, animal immunity, strain, and the amount of exposure to FMDV. Clinical signs of FMD are more severe in cattle kept in high-density pens (OIE, 2022). The clinical signs that characterize FMD seen in infected animals cannot yet be used as a diagnostic tool because several other infectious animal diseases have similar clinical signs resembling FMD. Several diseases with clinical symptoms similar to FMD include Swine Vesicular Disease (SVD), Vesicular Stomatitis, and Vesicular Exanthema (Wong *et al.,* 2020). Clinical signs only suggest that the animal is infected with FMDV, so laboratory testing is necessary to confirm the diagnosis (Adjid, 2020).

The RT-qPCR method is used as a diagnostic method for detecting FMDV because it has high sensitivity and specificity (Callahan *et al.,* 2002; El-Bagoury *et al.,* 2022). The RT-qPCR method with specific primers and fluorogenic probes was designed to target the 3D gene, a highly conserved region in the FMDV genome. The 3D protein is a non-structural protein that is very important for the replication and pathogenesis of FMDV (Callahan *et al.,* 2002). The 3D protein is an RNA polymerase that is very important for the replication of FMDV genome. The results of RT-qPCR in this study showed that 46.15% of samples were positive for FMDV, which is in line with the clinical signs shown by the affected cattle. The results of Nishi *et al*. (2019) using the RT-qPCR method with primers targeting the 3D gene were able to detect more positive samples for FMDV compared to the 5'UTR gene. This shows that RT-qPCR testing targeting the 3D gene has a higher sensitivity for clinical specimens from infected animals, so this method is suitable for diagnosing FMD.

FMDV has a diversity of serotypes and topotypes, so it is necessary to update the data to take into account new strains and lineages that may arise in different

Table 5. Genetic distance based on the VP1 gene of foot and mouth desease virus in this research were compared with reference isolates from GenBank

No.	Isolate name/ FMDV strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	O1/Manisa/TUR/69 (AY593823)																									
2	O/LAO/2/2006 (EU667451)	0.11																								
3	O/IRN/31/2009 (KY091284)	0.12	0.08																							
4	O/IRN/18/2010 (KY091283)	0.14	0.11	0.05																						
5	O/IRN/88/2009 (KY091282)	0.13	0.11	0.06	0.07																					
6	O/PAK/16/2010 (KY091285)	0.13	0.09	0.05	0.06	0.07																				
7	O/KUW/3/97 (DQ164904)	0.13	0.09	0.09	0.10	0.11	0.09																			
8	O/OMN/7/2001 (DQ164941)	0.12	0.10	0.09	0.11	0.11	0.10	0.04																		
9	O/UAE/4/2008 (KM921876)	0.13	0.10	0.10	0.12	0.11	0.12	0.06	0.07																	
10	O/BHU/3/2009 (KM921814)	0.13	0.11	0.11	0.12	0.12	0.12	0.07	0.07	0.09																
11	O/ISA/1/2022 (OP585403)	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.09															
12	O/ISA/HSU/A0522099-5/2022 (ON854957)	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.09	0.00														
13	O/ISA/Magetan/A04222620/2022 (ON854956)	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.09	0.00	0.00													
14	O/ISA/Semarang/A04222614/2022 (ON854955)	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.08	0.01	0.01	0.01												
15	O/ISA/Pemalang/A04222613/2022 (ON854954)	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.09	0.00	0.00	0.00	0.01											
16	O/CAM30/2019 (MZ634456)	0.15	0.14	0.12	0.14	0.13	0.13	0.10	0.10	0.11	0.07	0.06	0.06	0.06	0.06	0.06										
17	O/HKN/21/70 (AJ294911)	0.18	0.19	0.19	0.20	0.20	0.21	0.22	0.22	0.22	0.21	0.20	0.20	0.20	0.20	0.20	0.20									
18	O/TAI/189/87 (KY091288)	0.15	0.14	0.14	0.14	0.15	0.13	0.16	0.16	0.17	0.15	0.18	0.18	0.18	0.18	0.18	0.19	0.21								
19	C/N65/Tadjikistan/USSR/67 (KY091302)	0.49	0.49	0.49	0.49	0.50	0.47	0.48	0.47	0.45	0.46	0.48	0.48	0.48	0.48	0.48	0.47	0.50	0.49							
20	Asia1/PAK/1/54 (AY593795)	0.43	0.42	0.43	0.41	0.42	0.42	0.41	0.41	0.42	0.40	0.40	0.40	0.40	0.40	0.40	0.41	0.43	0.43	0.45						
21	O/ISA/MHW32-POCattle/2022 (OR570805)*	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.09	0.00	0.00	0.00	0.01	0.00	0.06	0.20	0.18	0.48	0.40					
22	O/ISA/MHW84-POCattle/2022 (OR570806)*	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.09	0.00	0.00	0.00	0.01	0.00	0.06	0.20	0.18	0.48	0.40	0.00				
23	O/ISA/MHW87-POCattle/2022 (OR570807)*	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.12	0.09	0.00	0.00	0.00	0.01	0.00	0.06	0.20	0.18	0.48	0.40	0.00	0.00			
24	O/ISA/MHW145-MaduraCattle/2022 (OR570808)*	0.13	0.13	0.12	0.13	0.12	0.13	0.11	0.11	0.11	0.09	0.01	0.01	0.01	0.01	0.01	0.06	0.21	0.18	0.47	0.41	0.01	0.01	0.01		
25	O/ISA/MHW311-MaduraCattle/2022 (OR570809)*	0.14	0.13	0.12	0.13	0.12	0.13	0.11	0.10	0.11	0.08	0.00	0.00	0.00	0.01	0.00	0.06	0.21	0.18	0.48	0.41	0.01	0.01	0.01	0.00	

Note: * (asterisk)= samples.

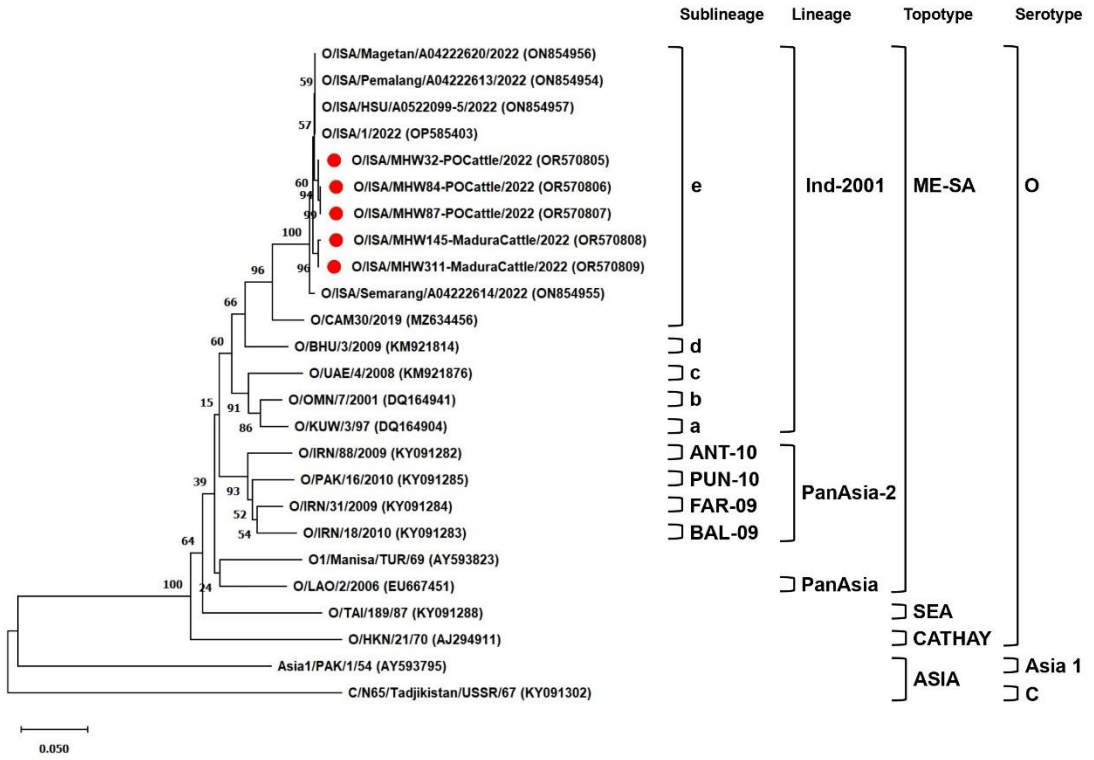

Figure 4. A phylogenetic tree was constructed by the Neighbor-Joining method with 1000 bootstrap replicates and the Kimura two-Parameter model. The phylogenetic relationships of five samples (marked with red circles ●) were compared with reference isolates from the GenBank. Sample code O/ISA/MHW32, O/ISA/MHW84, and O/ISA/MHW87 are Ongole Grade cattle, while sample code O/ISA/MHW145 and O/ISA/MHW311 are Madura cattle.

regions of the world (Knowles *et al.*, 2016). In this study, identification and molecular characterization based on amplification of the VP1 gene of FMDV were conducted to provide information about serotype prediction, virus clustering, and add molecular scientific data on FMDV in Indonesia. The RT-PCR method based on VP1 gene amplification is currently the ideal method for the detection of FMDV. The RT-PCR method is a fast and efficient method to obtain a greater fragment of the VP1 gene, which is suitable for direct sequencing, cloning, and molecular epidemiological studies based on the VP1 gene sequence without the need for cell culture or virus purification (Reid *et al.*, 2001). The important role of VP1 in virus attachment makes the nucleotide sequence of the VP1 coding region widely used to detect the characterization of FMDV strains. The VP1 protein is exposed on the surface of the virus capsid and is the main protein that determines the serotype and genotype of FMDV (Liu *et al.*, 2017).

Knowles & Samuel (2003) stated that RT-PCR is very effective as a method for diagnostic confirmation of FMDV. Samples with positive RT-PCR results in this study had low C_T values, namely MHW32 (30.38), MHW84 (31.17), MHW87 (29.86), MHW145 (27.32), and MHW311 (26.70). The C_T value is the number of cycles required for the fluorescent signal to exceed or cross the threshold line. The C_T value is inversely proportional to the amount of virus in the sample, a lower C_T value indicates a higher virus load (Cao *et al.*, 2020).

The results of this study showed that the arginine-glycine-aspartic acid (RGD) motif at positions 145–147 for receptor binding proteins was conserved across all samples included in the alignment, and no changes were found in critical amino acid substitutions at positions 144 (valine), 148 (leucine), 154 (lysine), and 208 (proline) in the VP1 protein epitope (amino acids 140–160 and 200–213). Changes also did not occur in the amino acid sites 145–147, which consist of arginine-glycine-aspartic acid (RGD), which plays a role in virus adsorption to host cells, similar to research conducted by Jinding *et al*. (2006). Residues 140–160 in the VP1 protein have been shown to induce neutralizing antibodies against FMDV types O and A (Wang *et al.*, 2007). The VP1 protein produces neutralizing antibodies in the infected animals at amino acid positions around 140–160 and 200–213, which are the most immunogenic regions (Qiu *et al.*, 2021). The VP1 protein consists of two important immunogenic sites, namely the G-H loop (amino acids 140–160) and the C-terminus (amino acids 200–213), where the G-H loop contains the RGD motif, which is required for viral attachment to host cells via integrin receptors (Jamal & Belsham, 2013) and stimulation of protective immune responses in the host (Fernandez-Sainz *et al.*, 2019). Single amino acid replacements in FMDV VP1, particularly in the vicinity of the RGD motif, may be involved in virus replication, pathogenicity (Bai *et al.*, 2019; Lian *et al.*, 2016), and receptor recognition (Bai *et al.*, 2014).

The results of the amino acid sequence analysis in this study showed that there were differences in the amino acid sequence of the VP1 protein between samples collected from Madura cattle and Ongole Grade

cattle. The three isolates O/ISA/MHW32-POCattle/2022, O/ISA/MHW84-POCattle/2022, and O/ISA/MHW87-POCattle/2022 do not have amino acid differences, but when compared with the other two isolates, O/ISA/MHW145-MaduraCattle/2022 and O/ISA/MHW311-MaduraCattle/2022 have different amino acids found at position 158. Amino acid variations were detected in amino acid residues A96T, L99E, V129A, S134C, D138E, T140A, A156T, A158T, and D197E in almost all positive samples in this study except for isolates O/ISA/MHW145-MaduraCattle/2022 and O/ISA/MHW311-MaduraCattle/2022, which do not have the A158T amino acid residue. Research conducted by Sheikh *et al*. (2021) regarding the first molecular characterization of serotype O in Iraq targeting the VP1 gene of FMDV shows that there is a change in critical amino acid substitutions in the G-H loop of the VP1 protein at positions 134–160, including D138E, G139S, T140R, V141A, A144T, and A158T, which are responsible for antigenic heterogeneity.

The value of genetic distance in the VP1 gene between samples in this study was between 0%–1%, with a homology value of between 99%–100%. The genetic distance between the sample and the Indonesian isolate studied in 2022 is 0%–1% with a homology value of 99%–100%. Isolate O1/Manisa/TUR/69 (AY593823) from Turkey had a genetic distance to the sample of between 13% and 14% with a homology value of 86%–87%. Isolate C/N65/Tadjikistan/USSR/67 (KY091302) from Tajikistan had a genetic distance to the sample of between 47% and 48% with a homology value of 52%–53%. The isolate Asia1/PAK/1/54 (AY593795) from Pakistan had a genetic distance to the sample of between 40% and 41% with a homology value of 59%–60%.

In the phylogenetic analysis in this study, samples were compared with reference isolates from the GenBank database of FMDV serotypes, including O, C, and Asia 1. Phylogenetic analysis based on VP1 sequencing has also been used to identify epidemiological relationships between genetic lineages, track original strains, and track the movement of outbreak cases (Jamal & Belsham, 2013). Phylogenetic tree analysis of the coding region of the VP1 gene in this study characterized the sequences of the five samples as members of the O/ME-SA/Ind-2001e lineage. The phylogenic tree shows that the samples are closely related to isolates from Indonesia that were previously reported by Susila *et al*. (2023) and Zainuddin *et al*. (2023). Apart from that, the sample is also closely related to the isolate from Cambodia, namely O/CAM30/2019 (MZ634456). Livestock traffic and trade in livestock products are the biggest risk factors in the spread of FMD between countries in the SEA (Blacksell *et al*., 2019).

CONCLUSION

FMD characterized by hypersalivation, lesions on the coronary band, nose, gums, and hard palate, and vesicles on the dental pads, gums, and feet in the affected animals were confirmed through molecular detection. Based on phylogenetic analysis of VP1 coding sequences, it was revealed that FMDV in this study sample belonged to the O/ME-SA/Ind-2001e lineage, which is serotype "O", topotype "Middle East South Asia (ME-SA)", lineage "Ind-2001", and sublineage "e".

CONFLICT OF INTEREST

We certify that there is no conflict of interest in any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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